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Fungal ecological strategies reflected in gene transcription – a case study of two litter decomposers

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Running title

Fungal ecology reflected in gene transcription

Originality-Significance Statement

The direct assessment of ecophysiological processes, such as decomposition of organic matter, is a key to understand the role of microbial communities in their environment. We addressed this challenge by comparing information given by genomes *vs.* transcriptomes of two fungi during a litter decomposition experiment, in parallel with a measure of organic matter chemical changes. Our findings highlight that contrasting ecological strategies were reflected by differences in expression of specific functional genetic markers, as well as temporal changes in gene expression of different components of the decomposer machinery, following the chemical changes in the substrate as decomposition progressed. Consequently, we assume that targeting transcription ratios of

specific ‘keystone’ genes would be useful to assess fungal ecological strategies, providing information about the dynamics of ecophysiological processes, such as decomposition, at the ecosystem scale.

Summary

Microbial communities interplay with their environment through their functional traits that can be as response or an effect to the environment. Here we explore how a functional trait – the decomposition of organic matter, can be address based on genetic markers and how the expression of these markers reflect ecological strategies of two fungal litter decomposer *Gymnopus androsaceus* and *Chalara longipes*. We sequenced the genomes of these two fungi, as well as their transcriptomes at different steps of Pinus sylvestris needles decomposition in microcosms. Our results highlighted that if the gene content of the two species could indicate similar potential decomposition abilities, the expression levels of specific gene families belonging to the glycosyl hydrolases (GH) superfamily reflected contrasting ecological strategies. Actually C. longipes, the weaker decomposer in this experiment, turned-out to have a high content of genes involved in holocellulose decomposition but low expression levels, reflecting a versatile ecology compare to the more competitive G. androsaceus with high expression levels of keystone functional genes. Thus we established that sequential expression of genes coding for different components of the decomposer machinery indicated adaptation to chemical changes in the substrate as decomposition progressed.

Keywords

Fungi, genomics/functional genomics/comparative genomics, Microbial ecology, Transcriptomics, Ecophysiology

Introduction

To understand relationships between composition and function of microbial communities it is essential to analyse how contrasting ecological traits and strategies interplay with the environment (Koide *et al.*, 2014). Direct assessment of ecophysiological traits of microorganisms colonizing natural substrates is a major challenge, but indirect approaches based on gene content and expression offer new ways forward. Here we explore how fungal ecological strategies can be assessed based on genetic markers of functional traits (Kuske *et al.*, 2015; Treseder and Lennon, 2015), using two fungal decomposers of needle litter with contrasting ecological strategies as a model.

During the past decades the number of sequenced fungal genomes has increased rapidly, especially due to the Community Science Program “1000 fungal genomes” launched by JGI (Grigoriev *et al.*, 2014). Genomic information enables comparative analyses of fungal species, in order to explain functional differences related to the content of genes with specific functions among genomes (Eastwood *et al.*, 2011; Floudas *et al.*, 2012; Talbot *et al.*, 2015; Martino *et al.*, 2018). Organic matter decomposition is a good example where losses (Kohler *et al.*,

2015) and gains (Floudas *et al.*, 2012; Riley *et al.*, 2014) of plant cell-wall degrading enzymes have been connected to the evolution of specialized ecological groups. Divergent evolution of the genetic machinery of decomposition has led to the distinction of fungal guilds within the general guild of saprotrophs, such as the white-rot fungi capable of lignin degradation (Floudas *et al.*, 2012). Consideration of different guilds of decomposers (*e.g.* opportunists, holocellulose decomposers and lignin decomposers) may enable better prediction of relationships between soil fungal communities, the chemical nature of organic matter and the loss and stabilization of organic pools below ground (Moorhead and Sinsabaugh, 2006; Talbot *et al.*, 2015; Bhatnagar *et al.*, 2018).

However, in relation to bacteria, fungi represent a narrow evolutionary branch, and it seems plausible that ecological strategies among fungi largely reflect differences in gene regulation rather than gene content. All genes involved in a process may not be of the same importance, and ecological strategies could be characterized by expression of certain keystone genes. Further, litter decomposition is biochemically sequential, and a transcriptomic approach can provide information about the genes expressed during different phases of the decomposition process. Fungal gene expression in response to the chemical composition of organic matter (Baldrian and López-Mondéjar, 2014), but also the subsequent alteration of organic matter by fungal gene products should be essential in defining the ecological strategy of fungal decomposers.

Fungal genes coding for enzymes involved in biochemical transformations during decomposition are specified according to the CAZyme classification (Lombard *et*

al., 2014). The CAZy database characterizes evolutionary distinct enzyme families with respect to their biochemical properties and substrate specificities. Enzymatic degradation during litter decomposition can be divided into three main processes linked to the principal components of plant cell walls:

(1) **Cellulose** decomposition is a complex process resulting from the action of a variety of glycoside hydrolases (GH) (Table1). Cellulases (primarily GH5-5, GH6 and GH7) cleave cellulose chains, decreasing their length and creating new chain ends. These enzyme can act on crystalline cellulose and may either be binding-releasing enzymes that have to attach to the cellulose via CBM for each hydrolytic cleavage or processive (non-releasing) enzymes that generate the dimer cellobiose (cellobiohydrolases) (Payne *et al.*, 2015). The 1,4-β-glucosidases (GH1 and GH3) then are able to hydrolyze cellobiose into glucose. There is also an additional, oxidative mechanism to cleave the cellulose internally, based on lytic polysaccharide monooxygenases (LPMO), which belong the Auxiliary Activities (primarily AA9) class of enzyme in the CAZyme database (Levasseur *et al.*, 2013).

(2) **Hemicelluloses** are branched polymers that, in addition to glucose, also contain fucose, galactose, rhamnose, mannose, arabinose and xylose (Sarkar *et al.*, 2009; Schädel *et al.*, 2010). Together these sugars form the three main polysaccharides of hemicelluloses: xylan, xyloglucan and galactomannan. Due to the molecular complexity of hemicelluloses, several sets of enzymes are involved in their decomposition (Table1). The involved gene families are polyspecific and contain enzymes that potentially may target many different substrates. As for cellulose, hemicellulose decomposition is initiated by endohydrolytic enzymes that

create new chain ends, enabling the subsequent action of processive exohydrolases followed by monomer-releasing enzymes. **Pectins** are also heteropolysaccharides that are degraded in a similar manner as hemicelluloses, with pectin lyases playing a key role.

(3) **Lignin** is a phenolic polymer that is resistant to hydrolytic decomposition. Breakdown of lignin, as well as other non-hydrolysable components, require oxidative mechanisms. In particular, enzymes in the class II peroxidase family (AA2) are able to attack a variety of chemical bonds in an unspecific manner. Class II peroxidases include lignin peroxidases , manganese peroxidases (MnP) and versatile peroxidases (Martinez *et al.*, 2009; Hofrichter *et al.*, 2010). Of these, MnP are the most abundant in soils (Kellner *et al.*, 2014) and act indirectly by oxidizing Mn^{2+} to Mn^{3+}, using H_2O_2 as electron acceptor. The Mn^{3+}, in turn, may oxidise a variety of organic molecules. Multicopper oxidases (AA1), including laccases, use molecular oxygen as electron acceptor and have also been proposed to participate in lignin oxidation.

The primary steps of decomposition are extracellular processes responsible for the depolymerisation of long polymers into low molecular weight compounds that may be taken up by the fungi and used in intracellular metabolism. Part of the acquired resources will then be used in catabolism to eventually be released in the form of CO_2 during respiration via two steps of the citric acid cycle mediated by isocitrate dehydrogenase (IDH) and the oxoglutarate dehydrogenase complex (OGDC). Remaining resources may be used for anabolic purposes, building cell components – primarily cell walls. The fungal cell wall is essentially composed by

two polymers; β-1,3-glucan, polymerized by glucan synthase (GT48), and chitin, polymerized by chitin synthase (GT2) (Bowman and Free, 2006). The carbon (C) use efficiency represents the proportion of acquired carbon that the organisms use to build biomass (Manzoni *et al.*, 2018).

In this study we addressed how changes in chemical composition of *Pinus sylvestris* needle litter were related to gene content and transcription during decomposition by two litter decomposer fungi with contrasting ecological strategies. We used the experimental material presented in Baskaran *et al.* (2019) and chemical data therein, derived from ^{13}C-PMAS-NMR spectroscopic analyses. *Gymnopus androsaceus* (L.) Della Magg. & Trassin. and *Chalara longipes* (Preuss) Cooke. are both common colonizers of pine litter but with different ecological strategies and abilities regarding decomposition (Baskaran *et al.*, 2019). *G. androsaceus* is an efficient decomposer with high ligninolytic capacity (Boberg *et al.*, 2011), whereas *C. longipes* is characterized by stress tolerance, endophytic capacity (Koukol, 2011) and lack of ligninolytic ability (Boberg *et al.*, 2011). Baskaran *et al.* (2019) found that in the presence of *G. androsaceus,* 40% of the needle litter mass was lost during 10 months of incubation, but only 10 % in the presence of *C. longipes*. Further, *G. androsaceus* was able to decompose non-hydrolysable constituents of the litter, including aromatic and akyl C, whereas *C. longipes* only exploited the hydrolysable litter fraction (mainly polysaccharide O-Alkyl C).

Here, we sequenced the genomes of the two fungi and analysed gene expression during decomposition. We expected that the differences in decomposer capacity

and ecology would be reflected in the genomes, in the expression of genes central for the decomposition machinery, as well as in the expression of genetic markers related to C use efficiency. C use efficiency should decrease with time, due to increased costs of mycelial maintenance and the more complex decomposing machinery required as the substrate become increasingly recalcitrant (Manzoni *et al.*, 2018). Further, C use efficiency was expected to be lower for *C. longipes*, as its stress-tolerant mycelium should have a higher cost of maintenance relative to its slow growth, compared with the rapidly growing *G. androsaceus* (c.f. the C-S-R ecological strategies of Grime 1974 (Cooke and Rayner, 1984)).

We hypothesized that:

(1) In the genomes, there would be a correlative link between the presence or number of genes with a specific function, the biochemical action of a fungus and its affiliation to a functional guild. While the gene diversity among relevant GH families would be predictors of cellulose, hemicellulose and pectin decomposition, presence of the AA1 and AA2 gene family should be indicative of lignin decomposition.

(2) In the transcriptomes, genes coding for lignocellulolytic enzymes would be expressed in sequence during the progressive decomposition of hemicellulose, cellulose and lignin. The relative expression of genes coding for hydrolases acting on long-chain substrates, including endohydrolases and processive enzymes as well as the oxidative AA9) would be high initially, promoting subsequent higher expression of genes coding for enzymes that release monosaccharides. Oxidative enzymes (AA1 and AA2)

would primarily be expressed at late decomposition stages (Šnajdr *et al.*, 2011).

(3) We expected that *G. androsaceus*, growing and decomposing more rapidly, would have a higher CUE than the slowly growing *C. longipes* (Manzoni *et al.*, 2018) and hypothesised that this difference would be reflected in a higher expression ratio of the anabolic enzymes GT48 and GT2 over the catabolic enzymes IDH and OGDC. Further, we expected that CUE (as indicated by the GT48 and GT2 to IDH and OGDC ratio) would decrease with time and increasing recalcitrance of the substrate.

Materials and methods

Fungal strains and microcosms

Strains of *Gymnopus androsaceus* (isolate JB14) and *Chalara longipes* (isolate BDJ) were obtained from the culture collection of the Department of Forest Mycology and Plant Pathology at the Swedish University of Agricultural Sciences. Details of the microcosm design are described in Baskaran *et al.* (2019). Briefly, a total of 24 microcosms were filled with 85g of sand and 11g of dry Scots pine needles (*Pinus sylvestris*) each and sterilised by gamma radiation. The needle litter contained 0.42% N, 50.6% C, had a C:N-ratio of 120 and a lignin concentration of approximately 25% (Boberg *et al.*, 2014). For each fungal species, twelve microcosms were inoculated through the addition of single needles that had been pre-colonized for 2 months on N-free agar cultures. After inoculation, 20 ml of 100 mM NH_4Cl solution were added to all microcosms, to stimulate fungal growth. Sealed microcosms were incubated at 20 ºC and 4

replicates from each fungal species were harvested after 2, 5 and 10 months, shock frozen in liquid nitrogen, ground using mortar and pestle and stored at -80 ºC.

A Bruker 500 MHz Avance III spectrometer equipped with a MAS probe was used to acquire ^{13}CP/MAS spectra (Schaefer and Stejskal, 1976), which were divided into established spectral regions to quantify the relative contribution of O-Alkyl C, Alkyl C, Aromatic C and Carboxyl C.

DNA and RNA extraction for genome sequencing, assembly and annotation.

C. longipes BDJ was cultured in liquid Hagem medium for 30 days and *G. androsaceus* JB14 was cultured in liquid medium with 1.75% malt and 0.25% peptone for 16 days. Mycelium was freeze-dried and ground in a mortar and pestle with sand, and 1-5 mL was used for DNA and RNA extraction. DNA was extracted following a 3% CTAB-buffer/chloroform protocol (supplementary method A). RNA was extracted with 2% CTAB-buffer/chloroform-isoamyl alcohol (supplementary method B) and purified following the protocol of the RNeasy mini kit (Qiagen).

G. androsaceus (https://genome.jgi.doe.gov/Gyman1/Gyman1.home.html) and *C. longipes* (https://genome.jgi.doe.gov/Chalo1/Chalo1.info.html) were sequenced by the U.S. Department of Energy Joint Genome Institute (JGI) using a combination of Illumina fragment (270 bp insert size) and 4 Kbp long mate-pair (LMP) libraries, and assembled using ALLPATHS-LG. *G. androsaceus* was improved with PacBio and PBJelly. The genomes were annotated using the JGI annotation pipeline (Grigoriev *et al.*, 2014) and are available via the JGI MycoCosm database

(jgi.doe.gov/fungi). Transcriptomes of the two species produced at JGI were sequenced using Illumina, assembled using Rnnotator and used for genome annotation (Kohler *et al.*, 2015).

RNA extraction from microcosm, sequencing and data processing

Total RNA was extracted from 800 mg of tissue per sample using the RNA PowerSoil Total RNA Isolation Kit (Mobio now Qiagen). Quantification and integrity check were conducted using an Experion Automated Electrophoresis Station (Bio-Rad, Hercules, CA, USA). Preparation of libraries (IlluminaTruSeq Stranded mRNA) and 2 x 125 bp Illumina HiSeq2500 sequencing was performed by the GeT platform (Get-PlaGe GenoToul, Castanet-Tolosan, France) following their standard protocol. Three replicates were sequenced except for *C. longipes* mycelium harvested after 2 and 5 month. For these two time points the amount of fungal material was only sufficient to extract good quality RNA for two replicates. Raw reads were trimmed for low quality (quality score 0.05), Illumina adapters and sequences shorter than 15 nucleotides and aligned to the respective reference transcripts available at JGI (https://genome.jgi.doe.gov/programs/fungi/index.jsf) using the CLC Genomics Workbench v9. The following CLC genomic workbench parameters were used for read mapping: minimum length fraction 0.9, minimum similarity fraction 0.8, Mismatch cost = 2, insertion cost = 3, Deletion cost = 3, and the maximum number of hits for a read was set to 10. The unique and total mapped reads number for each transcript were determined and then normalized to RPKM (Reads Per Kilobase of exon model per Million mapped reads). Intact pairs were counted as two, broken pairs as one. The complete data sets were submitted to NCBI GEO as GSEXXX.

Analysis of gene expression in microcosms

Specific gene families were selected from the CAZyme database (Lombard et al., 2014) or based on literature (Barbi *et al.*, 2014; Treseder and Lennon, 2015) In the specific cases of the GH2 family, only beta-mannosidases were selected based on JGI annotations (Table1). The number of genes in each selected gene family were integrated across the two fungal genomes, and the normalized number (RPKM) of sequenced transcripts were assessed. Statistical analyses were performed using R (v3.3.1) with "vegan" packages (Oksanen *et al.*, 2019; R Core Team, 2019). Changes in global patterns of gene expression during the experiment were analysed by Principal Component Analysis (PCA) based on mean-normalized expression values. Patterns of gene expression were related to changes in chemical composition of the decomposing litter by fitting vectors of C fraction ratios (Table 2) to the PCA. Specific correlations between expression ratios of genes from specific families and time or C fraction ratios were analysed *post-hoc* by linear regression.

Results

The 89 Mbp genome of *G. androsaceus* contained 29375 genes, of which 409 (1.4%) were identified as belonging to different GH families, while the 52 Mbp genome of *C. longipes* contained a total of 19765 genes, of which 429 (2.2%) were identified as GH genes (https://genome.jgi.doe.gov/Gyman1/Gyman1.home.html, https://genome.jgi.doe.gov/Chalo1/Chalo1.home.html) (Table S1).

The genome of *C. longipes* contained a somewhat higher number of genes coding for β-glucosidases (GH1 and GH3) and cellulolytic enzymes (GH5_5, GH6, and GH7) as that of *G. androsaceus* (56 vs. 52) (Fig. 1A; Table S1). *C. longipes* had 93 genes potentially involved in xylan decomposition (GH10, GH11, GH115, GH27, GH3, GH35, GH36, GH43, GH51, GH54, GH62 and GH67), whereas *G. androsaceus* had only 64. A high number of genes involved in hemicellulose decomposition seems to be a common feature of Leotiomycetes, which in addition to litter saprotrophs, such as *C. longipes*, also include endophytes and species forming ericoid mycorrhiza (Fig. S1). *C. longipes* had 34 genes involved in galactomannan decomposition (GH5_7, GH5_31, GH27, GH26, GH35 and GH36), whereas *G. androsaceus* had only 24 genes. Both fungi contained a similar number of genes involved in xyloglucan decomposition (GH12, GH27, GH29, GH31, GH35, GH36, GH51, GH54, GH74 and GH95; 48 and 47 for *C. longipes* and *G. androsaceus*, respectively) (Fig. 1C; Fig S2; Table S1). *C. longipes* had 46 genes involved in pectin decomposition, whereas *G. androsaceus* had only 32 (Fig. 1E). Regarding lignin breakdown, *C. longipes* had 28 genes annotated as multicopper oxidases, whereas *G. androsaceus* had 32 (Fig. 1G). Moreover, no genes coding for versatile or lignin peroxidase were identified in the genomes of any of the fungi, but the genome of *G. androsaceus* contained 10 genes coding for class II peroxidases (AA2) that were further classified as Manganese Peroxidases. *C. longipes*, similar to other ascomycetes, had 4 class II peroxidases with missing key residues for Mn oxidation (ExxxE and D) and these have no conserved tryptophan residue (Fawal *et al.*, 2013). These enzymes have been classified as ascomycete class II peroxidases or as “generic peroxidases” (Floudas *et al.*, 2012).

Transcriptomes were successfully sequenced from 9 microcosms with *G. androsaceus* (3 from each harvest) and from 7 microcosms with *C. longipes* (2 x 2 months, 2 x 5 months and 3 x 10 months). In contrast to the lower number of GH genes in the genome of *G. androsaceus*, the overall expression of β-glucosidases and cellulase genes were 8 times higher for *G. androsaceus* than for *C. longipes* (Fig. 1B), and genes coding for hemicellulases and pectin lyases were also more highly expressed by *G. androsaceus* (Fig. 1D; Fig. 1F; Fig. S2). Among genes involved in cellulose depolymerisation, *C. longipes* had 1.3 times more transcripts coding for β-glucosidases (GH1 and GH3) than for cellulases (GH5_5, GH6 and GH7), whereas *G. androsaceus* had higher relative expression of cellulases, with an average GH1+3 to GH5_5+6+7 ratio of 0.17 (Fig. 1A).

Gene expression during decomposition

For *G. androsaceus,* changes in chemical composition of the litter followed a clear temporal dynamic (Fig. 2A) from month 2 (top left) to month 10 (middle right) (Baskaran *et al.*, 2019). The overall pattern of gene expression changed during the progression of the experiment, as indicated by the clustering of transcriptomes according to harvest time in the mean-normalized PCA, as well as by the significant correlation between mass remaining and the PCA ordination axes (P=0.016). Changes in gene transcription occurred in parallel with changes in chemical composition of the litter, as indicated by the significant correlations between different C fraction ratios, as analysed by NMR, and the PCA ordination axes (Aromatic C to O-Alkyl C, P=0.015; Alkyl C to O-Alkyl C, P=0.026; Carboxyl C to O-Alkyl C, P=0.032) (Fig. 2).

Expression of *G. androsaceus* genes coding for cellulolytic enzymes acting on long chains (GH5_5, GH6 and GH7) increased during the experiment, whereas the global expression level of β-glucosidase coding genes that release glucose (GH1 and GH3) was stable, and many β-glucosidase genes were more highly expressed at month 2 and 5 compare to month 10 (Fig 2B; Fig S3A). The ratio of expression between cellulases and β-glucosidases transcripts (i.e. the ratio of GH5_5+GH6+GH7 to GH1+GH3) increased significantly with time (Fig. 3A) (R^2=0.897, *P*=0.001) and was correlated with the Carboxyl C to O-Alkyl C ratio, which is an indicator of increasing decomposition (*G. androsaceus* R^2=0.787, *P*=0.001) (Fig. 3A).

Similarly, considering *G. androsaceus* genes coding for hemicellulases, we observed that genes coding for monosaccharide releasing enzymes were relatively more expressed during the early stages of the experiment, whereas genes coding for long-chain acting enzymes were more highly expressed at later stages (Fig. 2B and 2C). With the exception of one GH26 and one GH5_7, genes coding for enzymes targeting general hemicellulose substrates and galactomannan (i.e. GH2, GH26, GH27, GH35, GH5_7 and GH5_31) had their maximum of expression at early stages. Pectinase encoding genes (GH28, GH55, CE8 (CE=Carbohydrate Esterases) and PL1 (PL=Polysaccharide Lyases)) tended to be highly expressed at intermediate stages of decomposition (Fig. S6). On the contrary, enzymes targeting xylan and xyloglucan (i.e. GH10, GH11, GH12) were mainly expressed at later stages (Fig. 2D).

Most of the *G. androsaceus* AA9 genes were highly expressed at month 2 but less pronounced over time (Fig. 2B; Fig. 3C). AA2 genes had a maximum expression level at month 5 (Fig. 2B). Although the correlation between the ratio of Aromatic C to O-Alkyl C and the AA2 transcript expression level was only marginally significant (R^2=0.296, p-value=0.129) (Fig. S5A), there was a trend that the lignin to polysaccharides ratio decreased when transcription of AA2 genes was high (Fig. 3B). AA1 genes, coding for multicopper oxidases, were expressed throughout the experiment without a significant correlation (R^2=0.183, p-value=0.25) with substrate chemical composition (Fig. S5C).

For *C. longipes* directional changes in gene expression levels were less obvious (Fig. S4A; Fig. S6). Due to the lack of good replication for *C. longipes,* interpretation of gene expression dynamics is ambiguous. Actually, neither mass loss nor C fraction ratios were significantly correlated with the PCA ordination (*P>0.5)*. Nonetheless, it appeared that genes coding for long-chain acting enzymes (GH5_5, GH5_7, GH6, GH7, GH10, GH11) as well as LPMO (AA9) were relatively more highly expressed at later stages, whereas genes coding for monosaccharide releasing enzymes (GH1, GH3, GH2, GH27 and GH35) were more expressed at early stages (Fig. S4B and S4C; Fig. S3B). Moreover, *C. longipes* genes coding for ascomycetes class II peroxidases (AA2) were not significantly expressed, and AA1 expression was much lower than for G. androsaceus (Fig. 1H).

It was visually obvious that *G. androsaceus* grew more vividly than *C. longipes*. It also decomposed the organic matter four times more rapidly and the gradual

substitution of plant derived organic matter to organic matter derived from fungal mycelium was indicated by a gradual increase in the ratio of Alkyl C to O-Alkyl C in presence of *G. androsaceus* (Fig. 4) (Baskaran *et al.*, 2019). We used the expression ratio of genes coding for the GT48 and GT2 families (β-1,3-glucan synthase and chitin synthase) over the isocitrate dehydrogenase (IDH) and the oxoglutarate dehydrogenase complex (OGDC) (responsible for CO_2 production in the citric acid cycle) as an indicator of C use efficiency. This expression ratio was 3 times higher for *G. androsaceus* than for *C. longipes* and stable over the duration of the experiment (Fig 3D).

Discussion

We related gene content and transcriptional patterns to chemical changes during litter decomposition by two saprotrophic fungi with contrasting ecological strategies. Contrary to our presupposition, the presence or diversity of functional genes in the two genomes did not reflect the performance of the two fungi during decomposition. Although *C. longipes* was a weak decomposer of holocellulose compared to *G. androsaceus* (Baskaran *et al.*, 2019), we found that *C. longipes* had a higher number of transcribed genes coding for enzymes involved in cellulose, hemicellulose and pectin decomposition than *G. androsaceus* (Fig. 1; Fig. S1; Fig. S2). However, the expression levels for genes considered as markers for plant cell wall decomposition were considerably higher for *G. androsaceus* than for *C. longipes*, with an almost 8 times higher average expression of genes coding for cellulases and β-glucosidases and 9 times higher expression of hemicellulose genes, as well as 4.5 time higher expression of pectinase genes over the time course of the experiment (Fig. 1; Fig. S2). Thus, fungal gene

447 expression was a better predictor of cell-wall polysaccharides decomposition than
448 gene content, indicating that genomic information do not suffice to understand
449 functional differences between fungi of different ecological strategies.
450
451 In this context, one may consider the recent evolutionary history and larger
452 genomes of fungi relative to bacteria, which commonly have smaller genomes
453 that are highly optimized according to environmental selection pressure
454 (Martínez-Cano *et al.*, 2015). Compared to bacteria, fungi may be considered
455 metabolically similar, with sugars as their principal energy source. Thus,
456 ecological strategies among fungi primarily relate to differences in the way they
457 use extracellular processes and host interactions to acquire sugars. In some cases
458 systematic genomic differences have been identified, e.g. between mycorrhizal
459 and saprotrophic basidiomycetes (Kohler et al., 2015) or between different modes
460 of wood decomposition (Riley et al., 2014; Hori et al., 2018). In contrast, our
461 findings indicate that the distinct ecological strategies of two fungal litter
462 saprotrophs were largely regulated at the transcriptional level with a high degree
463 of genomic redundancy. Rather than indicating extensive decomposition
464 capabilities, the diverse set of genes involved in cell-wall polysaccharides
465 decomposition of *C. longipes* could reflect a high degree of ecological resilience
466 and flexibility. Generally, Leotiomycetes (to which *C. longipes* belongs) have a
467 broader tolerance to constrained nutrient availability and low pH than
468 Agaricomycetes (to which *G. androsaceus* belongs) (Sterkenburg *et al.*, 2015).
469 Further, *C. longipes* may colonise living needles as an endophyte (Koukol, 2011),
470 and this versatile ecology may be comparable to ericoid mycorrhizal
471 Leotiomycetes, which have been proposed to switch between biotrophism and

saprotrophism and also have a high content of genes involved in cell-wall polysaccharide decomposition (Martino *et al.*, 2018) (Fig. S1).

In contrast to the stress tolerant, versatile strategy of *C. longipes*, the potent decomposer capacity of the highly competitive *G. androsaceus* seems to be related to high expression of a limited number of keystone genes. Its single gene within the GH6 family, beneficial for cleavage of crystalline cellulose (Payne *et al.*, 2015), was highly transcribed at an almost 40 times higher rate than the three GH6 genes of *C. longipes*, suggesting a keystone role of GH6 transcription for cellulose decomposition (Fig. 1). The presence of 10 AA2 genes (MnP) in *G. androsaceus* confirms the pivotal role of extracellular peroxidases for overall plant cell-wall decomposition (Floudas *et al.*, 2012), particularly in boreal ecosystems (Kyaschenko *et al.*, 2017; Stendahl *et al.*, 2017). While *G. androsaceus* was able to cause significant mass loss of non-hydrolysable litter components, *C. longipes*, with only four non-expressed genes coding for “generic” ascomycete class II peroxidases and a low expression of AA1 genes (multicopper oxidases incl. laccases), was unable to attack this fraction (Baskaran *et al.*, 2019).

We hypothesized that genes coding for plant cell wall decomposing enzymes would be sequentially expressed as decomposition progressed, with long-chain acting enzymes initiating the process, monosaccharide-releasing enzymes increasing in relative importance with time, and oxidative enzymes primarily being produced at late stages of decomposition. On the contrary, we found that genes coding for enzymes acting on long chains (including the cellulases of GH6 and GH7 as well as endoxylanases, xyloglucan endoglucanases and

endomannanases belonging to the families GH10, GH11, GH12, GH5 and GH26) were most expressed towards the end of the experiment, whereas genes coding for monosaccharide-releasing β-glucosidases (GH1 and GH3), β-mannosidases (GH2), galactosidases (GH 27 and GH35) and pectinases (GH28, GH55, PL1, CE8) were expressed also during early stages of decomposition (Fig. 2, Fig. S3, Fig S6). For both fungi the expression ratio of cellulases (GH5_5, GH6 and GH7) to β-glucosidases (GH1 and GH3) increased over time with a higher ratio for *G. androsaceus* (Fig. 3A). Further, the cellulases to β-glucosidases expression ratio also increased as the Carboxyl C to O-Alkyl C ratio increased (Fig. 2; Fig. 3A), which indicates loss of polysaccharides in relation to more stable compounds. Thus, counterintuitively, production of long-chain acting enzymes seemed to increase as the pool of hydrolysable polysaccharides was depleted. These results partly agrees with the dynamics of enzyme activities in other decomposition experiments (Šnajdr *et al.*, 2011; Presley *et al.*, 2018).

Based on these observations, we conceptualise that the polysaccharides of the plant cell wall do not constitute a homogenous pool with respect to susceptibility to enzymatic hydrolysis, but rather a spectrum from long unbranched molecules to highly branched and cross-linked structures. Linear chains may be efficiently hydrolysed by a minimum of internal chain cleavages, followed by rapid depolymerisation by processive (non-releasing) enzymes, leading to ample production of small polysaccharides. These, in turn, require high activity of monosaccharide-releasing enzymes for sugars to become available for uptake. As susceptible substrates are depleted, the proportion of branched and cross-linked polysaccharides raises, and thus increasing the demand for internal cleavage and

chain-end formation. Further, cross-linking and branching efficiently disrupt the processive mechanism of non-releasing hydrolases, leading to a lower production of small polysaccharides (Yoshida *et al.*, 2008) (Fig. 5). Thus, the increasing expression of genes coding for long-chain active enzymes with time should not be interpreted as accelerating rate of decomposition, but rather as a response to decreasing availability of susceptible substrates and a lower output of products per enzymatic reaction event.

Another interpretation would be that crystalline cellulose – the primary target of enzymes in the GH6 and GH7 families – is less readily hydrolysed than amorphous cellulose and hemicellulose and thus remains until later stages of decomposition, motivating late expression of GH6 and GH7 encoding genes. However, this theory disagrees with our observations that genes coding for AA9 enzymes (LPMO) – instrumental for the initiation of degradation of crystalline cellulose (Hu *et al.*, 2014) – were expressed primarily during early stages (for *G. androsaceus*), whereas genes related to decomposition of xylan and xyloglucan – the main hemicelluloses of the secondary cell wall of softwood (Shrotri *et al.*, 2017) – were primarily expressed at later stages (Fig. 2D).

In light of our results we suggest that the expression ratio of GH5_5+GH6+GH7 to GH1+GH3 could be used as a marker of declining substrate quality, with low ratios indicating opportunistic use of more labile substrates, whereas a high ratio indicate more efficient resource utilization, also targeting more recalcitrant compounds.

In the case of *C. longipes,* which did not use oxidative mechanisms for ligninolysis, the Aromatic C to O-Alkyl C ratio increased with time (Fig S5B) as holocellulose was degraded. In contrast, in *G. androsaceus*, this ratio decreased concurrently with an increase in AA2 gene expression from month 2 to month 5 (Fig 3B). Although the correlation was not clearly significant, we observed a trend of lower Aromatic-C to O-Alkyl-C ratio when AA2 gene expression was higher (Fig S5A). This may indicate that, after initial consumption of susceptible polysaccharides, the proportion of lignin increases, and cross-linking between polysaccharides and lignin (Iiyama *et al.*, 1994) may impede hydrolytic depolymerisation (Yoshida *et al.*, 2008). This view is congruent with the increasing ratio of Aromatic C over O-Alkyl C in *C. longipes*, but with the ligninolytic *G. androsaceus* the chemical barrier to hydrolysis may have been counter-acted by peroxidases making new polysaccharides susceptible to depolymerisation. The relatively early expression of AA2 genes and many AA1 genes in *G. androsaceus* was in disagreement with our original hypothesis, as well as with the result of Šnajdr *et al.* (2011), who observed increasing Mn-peroxidase and laccase activity at late decomposition stages. Earlier expression of oxidative enzymes during decomposition of pine needles may be explained by the high proportion of O-Alkyl C in the non-hydrolysable pool (Baskaran *et al.*, 2019), potentially cross-linked to lignin (Iiyama *et al.*, 1994). Moreover, sequential patterns of decomposition observed in the field may also reflect the ecological succession of fungi during litter decomposition, in which initial decomposition by endophytic ascomycetes is replaced by the later action of basidiomycetes (Voříšková and Baldrian, 2013).

The lytic polysaccharide monooxygenases (LPMO) of the AA9 family are, supposedly, secreted during the early steps of the decomposition process (Couturier *et al.*, 2015) and act in synergy with cellulases to enhance their decomposition capacity (Harris *et al.*, 2010; Hu *et al.*, 2014; Couturier *et al.*, 2016). This idea is in agreement with our observations of *G. androsaceus*, where AA9 gene expression declined as decomposition progressed and the more efficient oxidative AA2 enzymes were induced (Fig. 2B; Fig. 3C). However, *C. longipes* without expressed AA2 coding genes and low AA1 gene expression was unable to attack lignin, and when susceptible polysaccharides were depleted, expression of AA9 increased (Fig. 3C; Fig. S4B).

After uptake of decomposition products, carbohydrates are partitioned between catabolic and anabolic metabolic pathways. The expression ratio of genes coding for enzymes involved in the synthesis of the two principal components of the fungal cell wall, namely β-1.3 glucan and chitin (i.e. GT48 and GT2) over genes coding for central enzymes of the respiration (i.e. IDH and OGDC in the Citric acid cycle) was much higher in *G. androsaceus* than *C. longipes* (Fig. 3D). This higher expression ratio is indicative of a higher CUE for *G. androsaceus*, in line with our initial hypothesis and the intense mycelial growth of *G. androsaceus* observed in the microcosms (Fig. 4). *C. longipes* had a similar expression of genes associated with CO_2 production, but lower expression of GT48 and GT2 genes, in line with its meagre mycelial growth. However, contrary to our hypothesis the gene expression indicator of CUE ratio remained constant over the experiment, indicating that the partitioning of acquired resources between growth and respiration was not altered by decreasing chemical quality of the substrate or

mycelial senescence. Thus, the increasing enzyme production in more recalcitrant materials, as indicated by the upregulated transcription of CAZyme genes with time, seems not to be associated with a major respiratory cost. However, the markers we used to reflect respiration are putative, and careful analyses of gene expression and CO_2 production in parallel are needed. Our observations may be interpreted within the C-S-R theoretical framework (Grime, 1977; Cooke and Rayner, 1984; Crowther et al. 2014), with *C. longipes* having stress-tolerant traits, such as a versatile but inefficient decomposition machinery and low resource allocation to mycelial growth. In contrast, the traits of *G. androsaceus* are congruent with a C-strategy, with higher biomass production and efficient exploitation of organic matter resources.

To conclude, expression analysis of specific genetic markers seems to be useful to assess fungal ecological strategies, providing information about the dynamics of ecophysiological processes, such as decomposition. By targeting transcription ratios of specific 'keystone' genes at the ecosystem scale (*i.e.* meta-transcriptomics), information about the interplay between the fungal communities and their environment may be derived that could be used to decipher the role of fungi as mediators of ecosystem responses to environmental change (Lindahl and Kuske, 2013; Treseder and Lennon, 2015).

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The authors have no conflict of interest to declare.

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Data accessibility

Gymnopus androsaceus genome: https://genome.jgi.doe.gov/Gyman1/Gyman1.home.html

GenBank accessions: BioProject PRJNA234428, BioSample SAMN05660848, Accession VKGB00000000.

Chalara longipes genome: https://genome.jgi.doe.gov/Chalo1/Chalo1.info.html

GenBank accessions: BioProject PRJNA213334, BioSample SAMN02745709, Accession VKGA00000000.

Microcosms experiment transcriptome data sets: NCBI GEO as GSEXXX *(Submission in progess)*

Author Contributions

FB, AKo, PB, BL, FM designed research; LF, PB, KI performed research; KI, LF, BH, IG contributed new reagents or analytical tools; AKu, KL, CD, KB, BH, IG were involved in genomes project and annotations; FB, AKo, EM, BL analyzed data; FB, AKo, BL, FM wrote the paper.

Table 1. Characteristics of the selected CAZyme gene families.

For each CAZyme family information concerning the enzyme substrate, mechanism, active site, 3D structure of catalytic domain and binding location are from the CAZyme database (http://www.cazy.org/), CAZypedia (http://www.cazypedia.org/index.php/Main_Page), ExplorEnz database (http://www.enzyme-database.org/index.php), and JGI annotation (https://jgi.doe.gov)

*GH2 beta-mannosidase were selected based on JGI annotations

**GH28 pectine lyases were selected based on JGI annotations

***This family include exo- and endo-enzymes but a majority of the member are exo-enzymes (CAZyme)

****Including subfamilies AA1_1, AA1_2 and AA1_3

Table 2. Interpretation of ^{13}C CP/MAS-NMR spectroscopy

Specific C pools of chemical fractions of *Pinus sylvestris* needle litter are interpreted in terms of organic matter compounds.

Figure 1. Gene content in genomes and global expression levels

(A, C, E and G) Bar plots indicating the number of genes present in the genomes of *Gymnopus androsaceus* and *Chalara longipes*. **(B, D, F and H)** Bar plots indicated the global expression levels (i.e. addition of the average of relative gene expression levels for each month). **(A and B)** Selected gene families involved in cellobiose and cellulose decomposition are indicated in light blue (GH1), blue (GH3), light green (GH5 (subfamily 5)), green (GH6), dark green (GH7). **(C and B)** Restricted number of relevant gene families coding for enzymes targeting a specific substrate in hemicelluloses are indicated in cyan (GH10), blue (GH11), purple (GH12), red (GH2), dark green (GH26), green (GH27), yellow (GH35) and brown (GH5 (subfamilies 7 and 31)). **(E and F)** Selected gene families involved in pectin decomposition are indicated in light blue (GH28*), darkblue (GH55), green (CE8), yellow (PL1), orange (PL3_2). **(G and H)** Selected gene families coding for

multicopper oxidases are indicated in brown (AA1), darkred (AA1_1), red (AA1_2), pink (AA1_3).

GH2*: Only beta-mannosidase. Based on JGI annotations. Transcript Id 916611, 962261 and 1012799 for *G. androsaceus*. JGI Transcript Id 193197, 345240, 354232, 396534, 406068, 470006, 493784 and 503773 for *C. longipes*.

GH28* For *G. androsaceus* the JGI Transcript Id 991351 is not annotated as pectinase.

Figure 2.

Principal component analysis (PCA) ordination displaying *Gymnopus androsaceus* overall pattern of gene expression during the experiment. Relative abundance of expressed genes are mean-normalized to represent the dynamic of gene expressions. Areas represent the global expression levels (i.e. sum of the average for each of the 3 month) for the selected genes, at a logarithmic scale. Vectors fitted the litter mass remaining and different carbon fractions of litter organic matter to the PCA ordination. The different plots highlight **(A)** the transcriptomes from month 2 (M2T1, M2T2, M2T3), month 5 (M5T1, M5T2, M5T3) and month 10 (M10T1, M10T2, M10T3), **(B)** expressed genes involved in cellobiose and cellulose decomposition (monosaccharide releasing enzymes in blue, long-chain acting enzymes in green and LPMO in red) and lignin decomposition (Class II peroxidases in purple), **(C)** expressed genes coding for hemicellulases (monosaccharide releasing enzymes in blue and long-chain acting enzymes in green), **(D)** the different hemicelluloses (xylan and xyloglucan in blue, galactomannan in green and all of them in red) targeted by the hemicellulases.

Figure 3.

(A) Relationship between the ratio GH5_5+GH6+GH7 to GH1+GH3 and the ratio Carboxyl C to O-Alkyl C. **(B)** Evolution over months of the relative abundance of expressed genes coding for class II peroxidases (AA2) in parallel with the ratio Aromatic C to O-Alkyl C in presence of *G. androsaceus* (purple). **(C)** Evolution over months of the relative abundance of expressed genes coding for lytic polysaccharide monooxygenases (AA9). **(D)** Relationship between the relative abundance of expressed genes coding for the β-glucan synthase (GT48) plus the chitin synthase (GT2) and the genes coding for the oxoglutarate dehydrogenase complex (OGDC) plus isocitrate dehydrogenases (IDH). Data obtained from litter decomposition microcosms in presence of *Gymnopus androsaceus* (blue) and *Chalara longipes* (red) at month 2 (empty circle), month 5 (circle with cross) and month 10 (full circle). Lines represent fitted linear regressions with $P < 0.05$ (solid line), $0.1 < P > 0.05$ (dashed line), $P > 0.1$ (dotted line).

Figure 4. Needle litter microcosms.

The pictures of the litter microcosms have been taken after 10 month in presence of *Gymnopus androsaceus* (left side, blue) and *Chalara longipes* (right side, red). The bar plots indicate the percentage of needle litter mass remaining and the gradual substitution of plant derived organic matter to fungal derived organic matter (i.e. ratio Alkyl C to O-Alkyl C) measured at month 2 (M2), 5 (M5) and 10 (M10) for both fungi.

Figure 5. Conceptual figure of plant polysaccharide pools hydrolysis over time. Plant cell wall constitutes a spectrum from long unbranched molecules to highly branched and cross-linked structures. **(Top frame)** At the beginning of the decomposition process, long unbranched molecules are efficiently hydrolysed by few long-chain acting enzymes and require high activity of monosaccharide-releasing enzymes for sugars to become available for uptake. **(Bottom frame)** In later stage of decomposition process, the higher proportion of branched and cross-linked structures increase the demand for internal cleavage and chain-end formation performed by long-chain acting enzymes an require equivalent or lower activity of monosaccharide-releasing enzymes. Consequently with the progress of decomposition the ratio of long-chain acting enzymes to monosaccharide-releasing enzymes increase.

Table S1. CAZy assignments for the 2 fungi *Gymnopus androsaceus* and *Chalara longipes*.

Table S2. *G. androsaceus* and *C. longipes* genomes information

Figure S1. Heatmap representing the degree of CAZYmes content similarity between *Chalara longipes*, *Gymnopus androsaceus* and 28 other Ascomycota and Basidiomycota published genomes. From left to right Cadospora sp. (Cadsp1) END, Oidodendron maius (Oidma1) ERM, Phialocephala scopiformis (Phisc1) END,

Chalara longipes (Chalo1) SAP, Meliniomyces variabilis (Melva1) ERM, Armillaria cepistipes (Armcep1) SAP, Gymnopus androsaceus (Gyman1) SAP, Gymnopus luxurians (Gymlu1) SAP, Amanita muscaria (Amamu1) ECM, Paxillus involutus (Paxin1) ECM, Laccaria bicolor (Lacbi2) ECM, Postia placenta (Pospl1) SAP, Serpula lacrimans (Serla2) SAP, Heterobasidion annosum (Hetan2) P, Trametes versicolor (Trave1) P, Phanerochaete chrysosporium (Phchr2) SAP, Schizophyllum commune (Schco3) SAP, Pleurotus ostreatus (Pleos2) SAP, Coprinopsis cinerea (Copci1) SAP, Sacharomyces cerevisiae (Sacce1) Y, Tuber melanosporum (Tubme1v2) ECM, Terfezia boudieri (Terbo2) ECM, Morchella importuna (Morco1) SAP, Neurospora crassa (Neucr2) SAP, Ascocoryne sarcoides (Ascsa1), Glarea lozoyensis (Glalo1) SAP, Glonium stellatum (Glost2) SAP, Rhizoscyphus ericae (Rhier1) ERM, Aspergillus nidulans (Aspni7) SAP, Cenococcum geophilum (Cenge3) ECM. Fungal guilds: ericoid mycorrhiza (ERM), endophyte (END), Ectomycorrhiza (ECM), saptrotroph (SAP), pathogen (P), yeast (Y).

Figure S2. Gene content in genomes and global expression levels

On the left: bar plots indicating the number of genes coding for hemicellulases involved in xylan, xyloglucan and galactomannan decomposition, present in the genomes of *Gymnopus androsaceus* and *Chalara longipes*. On the right: bar plots indicating the global expression levels (i.e. addition of the average of relative gene expression levels for each month) of genes coding for hemicellulases.

Figure S3. Dynamic of transcription for genes involved in cellobiose and cellulose depolymerisation.

Relative abundance of expressed genes coding for monosaccharide releasing enzymes (β-glucosidases GH1 in green and GH3 in yellow), genes coding for long-chain acting enzymes (GH5_5 in black, GH6 in blue and GH7 in red), at month 2, 5 and 10, for **(A)** *Gymnopus androsaceus* and **(B)** *Chalara longipes* (right). Lines represent fitted linear regressions with $P < 0.05$ (solid line), $0.1 < P > 0.05$ (dashed line), $P > 0.1$ (dotted line).

Figure S4.

Principal component analysis (PCA) ordination displaying *Chalara longipes* overall pattern of gene expression during the experiment. Relative abundance of expressed genes are mean-normalized to represent the dynamic of gene expressions. Areas represent the global expression levels (i.e. sum of the average for each of the 3 month) for the selected genes, at a logarithmic scale. Vectors fitted the litter mass remaining and different carbon fractions of litter organic matter to the PCA ordination. The different plots highlight **(A)** the transcriptomes from month 2 (M2T1, M2T2), month 5 (M5T1, M5T2) and month 10 (M10T1, M10T2, M10T3), **(B)** expressed genes involved in cellobiose and cellulose decomposition (monosaccharide releasing enzymes in blue, long-chain acting enzymes in green and LPMO in red), **(C)** expressed genes coding for hemicellulases (monosaccharide releasing enzymes in blue and long-chain acting enzymes in green), **(D)** the different hemicelluloses (xylan and xyloglucan in blue, galactomannan in green and all of them in red) targeted by the hemicellulases.

Figure S5.

(A) Relationship between the ratio Aromatic C to O-Alkyl C and the relative abundance of expressed genes coding for Class II peroxidases (AA2) for *Gymnopus androsaceus* at month 2 (empty circle), month 5 (circle with cross) and month 10 (full circle). **(B)** Evolution over month of the ratio Aromatic C to O-Alkyl C in presence of *Chalara longipes*. **(C)** Relationship between the relative abundance of expressed genes coding for multicopper oxidases (AA1+AA1_1+AA1_2+AA1_3) and the ratio Aromatic C to O-Alkyl C. Data obtained from litter decomposition microcosms in presence of *Gymnopus androsaceus* (blue) and *Chalara longipes* (red) at month 2 (empty circle), month 5 (circle with cross) and month 10 (full circle). Lines represent fitted linear regressions with $P < 0.05$ (solid line), $0.1 < P > 0.05$ (dashed line), $P > 0.1$ (dotted line).

Figure S6.

Principal component analysis (PCA) ordination displaying *Gymnopus androsaceus* and *Chalara longipes* overall pattern of gene expression during the experiment. Relative abundance of expressed genes are mean-normalized to represent the dynamic of gene expressions. Areas represent the global expression levels (i.e. sum of the average for each of the 3 month) for the selected genes, at a logarithmic scale. Vectors fitted the litter mass remaining and different carbon fractions of litter organic matter to the PCA ordination. The different plots highlight **(A)** *Gymnopus androsaceus* transcriptomes from month 2 (M2T1, M2T2),

990 month 5 (M5T1, M5T2) and month 10 (M10T1, M10T2, M10T3), **(B)** *Gymnopus*
991 *androsaceus* expressed genes involved in pectin decomposition (yellow and
992 green) and expressed genes coding for laccases (blue). **(C)** *Chalara longipes*
993 transcriptomes from month 2 (M2T1, M2T2), month 5 (M5T1, M5T2) and month 10
994 (M10T1, M10T2, M10T3), **(D)** *Chalara longipes* expressed genes involved in pectin
995 decomposition (yellow and green) and expressed genes coding for laccases (blue)
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